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*TRANSLATOR'S DECLARATION*

*I, Stephen V. Vitek, technical translator and manager of PatentTranslators.com, 1304 False Creek Way, Chesapeake, VA 23322, hereby certify that I am a technical translator fluent in the German and English languages, as well as a member in good standing of the American Translators Association, National Capital Area Translators Association, Northern California Translators Association, etc., and that my translation of PCT Patent Application No. WO2004/009223 A1 (MEMBRANE, FILTRATION MODULE AND METHOD FOR THE SEPARATION OF BIOMOLECULES FROM A LIQUID) is to the best of my knowledge and ability a true and faithful translation of this document in the German language into English.*

*Signed in Chesapeake, Virginia, on November 2, 2004.*

Stephen V. Vitek

**STEPHEN V. VITEK, TECHNICAL TRANSLATOR**

**Membrane, Filtration Module and Method for the Separation of Biomolecules from a Liquid**

The invention relates to a membrane comprising a microporous membrane body with an affinity ligand, which is capable of interacting with at least one type of molecules found in a fluid.

The invention further relates to a filtration module for the separation of biomolecules from a liquid, comprising a housing and at least one membrane.

The invention further also relates to a method for the separation of biomolecules from a liquid by means of membranes with chemically activated microporous membrane bodies to which affinity ligands are coupled, which are capable of interacting with the biomolecules to be separated.

Spherical carriers in the form of gel for affinity ligands have been employed for a long time in many spheres of biotechnology for purification and separation of a great number of different types of biomolecules. An example of this use is represented by affinity carriers based on agarose, which are commercially available in a suspension. The suspension medium can be in this case water or another solution means. Only a few matrices are offered in lyophilized form.

Furthermore, it is difficult or impossible to dry matrices once they have become swollen in an aqueous medium

because the small gel spheres are irreversibly damaged during this process. Preservation and transport of such gels thus presents a considerable logistical problem.

From EP 0 787 523 A1 it is known that ligands of a carrier material can be coupled for separation of substances having affinity. The function of the ligands is to bind a single target substance or even an entire class of substances which are specifically absorptive.

It is further known from DE 196 17 775 A1 that membrane adsorbers or membranes can be used carrying ligands which are capable of interacting with at least one substance being in contact in the liquid phase. The transport of the liquid phase through the membrane occurs in this case convectively due to a difference in pressure.

A disadvantage of the known separation of biomolecules is that drying of the carriers or the membranes with the coupled affinity ligand must be suppressed with a complicated procedure to prevent loss of the bioactivity of the ligands. Presence of water moisture in the membrane involves the risk of a microbial attack, and it also means that a conservation means will have to be added.

The goal of the present invention is therefore to provide membranes for separation of biomolecules from a liquid by means of affinity ligands enabling to prevent complicated and costly wet storage of the membranes.

This task is achieved in connection with the characterizing clause of claim 1 so that the membrane body can be stored dry with the affinity ligand, while the activity of the affinity ligand is retained.

Because the membrane body can thus be stored practically without a significant loss of activity, the storage and transportation costs can be significantly reduced and the separation of the biomolecules is simplified.

Surprisingly enough, it was found that membranes charged with affinity ligands, such as proteins, can be stored dry for a longer period of time without a loss of activity. This is applicable to microporous membranes made by the Sartorius AG Gőttingen Company, which are commercially available under the trade name of Sartobind®. The term "dry" should be understood as relating to membranes or membrane bodies whose pore volume is substantially filled with air. This does not exclude cases when the inner surface is covered with a highly volatile organic substance.

Suitable membranes are membranes having a microporous adsorptive body based for example on cellulose acetate (CA), cellulose nitrate (CN), polyamide, PESU, PP, PVDF. The size of the pores should be from 0.01 to 15 μm. A preferred pore size is in the range from 0.2 to 5 μm. The membrane body is further characterized by a thickness from 100 to 500 μm, preferably from 200 to 300 μm.

The membranes are preferably activated chemically, so that the affinity ligands can be chemically coupled. However, physical binding of the affinity ligands to the membrane is also possible.

According to a preferred embodiment form, the invention is equipped with a membrane body that is impregnated with glycerine. The impregnation with glycerine in this case also helps to prevent damage from being caused during the drying process

to the structure of the microporous membrane or of the membrane body.

Possible affinity ligands are known to a person in the art. As examples of adsorptive ligands are named:

- thiophiles,
- hydrophobes with different chain lengths and configurations,
- Reversed Phase,
- reactive pigments and other pigments,
- low-molecular non-charged or charged organic molecules,
- aminoacids and analogs,
- coenzymes, cofactors and analogs thereof,
- substrates and analogs thereof,
- endocrine and exocrine substances such as hormones and effectors having an effect similar to that of hormones and analogs thereof,
- enzyme substrates, enzyme inhibitors and analogs thereof,
- fatty acids, fatty acid derivatives, conjugated fatty acids and analogs thereof.
- nucleic acids
- DNA and analogs and derivatives thereof,
- RNA and analogs and derivatives thereof,
- monomers and analogs and derivatives thereof,
- oligopolymers including also polymers and analogs and derivatives thereof,
- high-molecular carbohydrates, linear or branched; unsubstituted or substituted,
- glycol conjugates, such as
    - heparin
    - amylose, cellulose
    - chitin, chitosan,
    - monomers and oligomers,
    - all derivatives and analogs thereof,
    - lignin and derivatives and analogs thereof.

- high-molecular ligands such as
  - proteins and oligomers thereof, multimeres, subunits and parts thereof,
  - peptides, polypeptides of analogs as well as derivatives thereof,
  - lectine,
  - antibodies and parts thereof,
  - fusion proteins,
  - haptenes,
- enzymes and subunits, as well as parts thereof,
- structural proteins,
- receptors and effectors, as well as parts thereof,
- xenobiotics
- pharmaceutics and pharmaceutically active substances
- alkaloids
- antibiotics
- biomimetics

Another task of the invention is to provide a device or a filtration module that is suitable for a cost-effective and efficient separation of biomolecules.

This additional task is solved according to the invention in connection with the characterizing clause of claim 9 so that the membrane is constructed according to one of the claims 1 through 8.

Thanks to the construction of the membrane according to one of the claims 1 through 8, the filtration module is provided with the advantages named above.

In particular, a selective separation of different biomolecules can be achieved by using a plurality of membranes. Moreover, the membranes can be also adjusted depending on the relevant separation problem in a relatively simple manner.

The membranes can be arranged in a housing in multiple layers in a housing. However, they can be also arranged serially in different housings or housing chambers.

The known methods for separation of biomolecules have the disadvantages named above.

That is why another task of the present invention is to provide an efficient and cost-effective method for separation of biomolecules from a liquid to be processed, which makes it possible to avoid complicated wet storage and transport.

This task is achieved in connection with the characterizing clause of claim 12 so that the following steps are taken:

a) Coupling of affinity ligands in a solution means to the membrane body,

b) Washing of the membrane body with at least one washing medium,

c) Extensive removal of the last washing medium with a drying step,

d) Intermediate dry storage of the membranes when this is required, and

e) Filtration of the liquid through the membranes, so that the biomolecules can be separated.

With the possibility of dry storage of the membrane without a loss of activity, both the storage and the transport are simplified and rendered less expensive.

In order to exclude as much as possible the risk of a microbial attack when water is used as a washing medium, the membrane is dried preferably to a water activity of 40%.

Under the term water activity is to be understood equilibrium partial pressure of water in relationship to pure water having the same temperature.
To the last washing medium according to step b) can be also added a strongly volatile organic substance or components that are miscible with the washing medium as an impregnation means. The impregnation means remains in the membrane during the drying stage. A film can be also formed on the surface of the pores or the membrane matrix can be obtained in a swollen status.

Other details of the invention will become obvious from the following detailed description and attached figures which illustrate preferred embodiment forms of the invention by way of examples.

Figure 1: A schematic illustration of a filter module with a membrane arranged in a housing,

Figure 2: a schematic illustration of a filter module for the separation biomolecules with membranes connected in series in a housing, and

Figure 3: a schematic illustration of a filter module for the separation of biomolecules with membranes arranged in several layers in a housing.

A filter module 1 for the separation of biomolecules from a liquid comprises essentially a housing 2 and a membrane 3 having a membrane body 4.

The housing 2 is provided with an inlet 5 and an outlet 6. The membrane 3 is equipped with its microporous adsorptive membrane body 4, which can be constructed for example on the basis of cellulose acetate, cellulose nitrate polyamide, PESU, PP, PVDF

with a pore size in the range from 0,01 to 15 μm, preferably 0.2 to 5 μm. The membrane body 4, which is provided with a planar construction, has a thickness in the range from 100 to 500 μm, preferably from 200 to 300 μm. To the membrane body 4 are coupled affinity ligands known to persons in the art (not shown in the figure). The affinity ligands are selected in such a way so that they have the capability to interact with the biomolecules to be separated from the processed liquid.

The membrane 3 can be provided as a single layer arranged in the housing 2 as shown in Figure 1. At the same time, several housings 2 that are provided with membranes 3 can be arranged in series.

However, it is also possible to arrange membranes 3' in several layers in one housing 2'. Affinity ligands, not shown in the figure, are chemically coupled to the membranes 3, 3', the membranes 3, 3' are impregnated with glycerine and then subjected to a drying process. Water is removed during this process to a very high degree from the membranes 3, 3'. After dry storage or after a transport, the liquid for processing is supplied through the inlet 5 to the membranes 3, 3' and transported convectively through the membranes, so that the liquid can be discharged through the outlet 6. At the same time, the biomolecules to be separated are bound to the affinity ligands.

Example:

The solution below referred to as PBS was prepared as described by J. Sambrook, E. F. Fritsch, T. Maniatis, in "Molecular Cloning" – A Laboratory Manual, second edition, Cold Spring Harbour Laboratory Press, 1989, Book 3, Appendix b. 12, in the following manner:

| g/l | Substance |
|---|---|
| 8.0 | Sodium chloride NaCl |
| 0.20 | Potassium chloride KCl |
| 1.44 | di-sodium hydrogenate phosphate $Na_2HPO_4$ |
| 0.24 | Potassium hydrogenate phosphate $KH_2PO_4$ |
| pH 7.4 ± 0.2 | |

A microporous membrane functionalized with aldehyde groups of the type Sartobind®, Aldehyde Membrane Code 19306, was reacted with protein A. For this purpose, protein A, manufactured by the Repligen Company, Designation rPrA, Lot No. 011038, was dissolved in 10 mg/ml of PBS. Three filter disks with a diameter of 25 mm were used in 2 ml of this solution, which was shaken in a small plastic Petri dish for 3 hours at ambient temperature. To reduce the Schiff bases created in this manner, 1% of the final concentration of cyanoborohydride was added. After the reaction took place, the membranes were removed and transferred to a fresh Petri dish. In order to reduce the remaining aldehyde groups, 5 ml of a solution of sodium borohydride in PBS with a final concentration of 1% was added and shaking was applied to the membranes for another 15 minutes. The membranes were then washed sequentially with PBS, using a solution of 0.1 M glycine, pH 2.7, 1 mM HCl, 1 mM NaOH and 1 mM NaCl in 0.01 M potassium phosphate, pH 7.0. The membranes were then dried at ambient temperature with an air current for 3 hours and stored at 4°C while air was substantially excluded.

The membranes were removed in predetermined time periods and tested with respect to their binding capacity for human immunoglobulin of the type IgGlund IgG2.

At the same time, the three membranes described above were built into a syringe adaptor with a diameter of 25 mm, Order No. 16517,

made by the Sartorius AG company which was equipped with a disposable syringe.

Processed human plasma from a local blood bank was diluted with PBS with a ratio of 1 : 40 and this solution was filtered through a 0.2 µm membrane. The syringe was filled with 10 ml of this solution obtained in this manner and filtration was carried out by using gravity through three built-in membranes. After that, washing was conducted with 10 ml of PBS and the bound amount of IgG was eluted with 10 ml of 0.1 M glycine, pH 2.7. The absorption of the elution solution was determined at 280 nm with a spectrum photometer and a manually adjustable calibration apparatus using bovine serum albumin as the comparison substance was used to determine the protein binding capacity.

The results of this test are shown in the table below.

*Table*: Time Variations of the IgG Binding Capacity of Protein A Charged with Aldehyde-Functionalized Membranes

| Time (Days) | Binding Capacity (µg/cm$^2$) |
|---|---|
| 0 | 42 |
| 1 | 41 |
| 4 | 47 |
| 20 | 43 |
| 45 | 40 |
| 56 | 37 |

All values are median values obtained with at least 2 measurements.

Patent Claims

1. A membrane comprising a microporous membrane body having an affinity ligand capable of interacting with at least one type of molecules found in a liquid, **characterized by the fact** that the membrane body (4, 4') can be stored with the affinity ligand, with retention of the activity of the ligand.

2. The membrane according to claim 1, **characterized by the fact** that the membrane body (4, 4') is chemically activated and the affinity ligands are chemically coupled.

3. The membrane according to claim 1, **characterized by the fact** that the affinity ligand is a biomolecule, whose specificity and/or capacity is substantially retained in dry status of the membrane body (4, 4').

4. The membrane according to one of the claims 1 through 3, **characterized by the fact that** the membrane body (4, 4') is provided with a pore size in the range of 0.1 to 15 µm.

5. The membrane according to one of the claims 1 though 3, **characterized by the fact** that the membrane body (4, 4') is provided with a pore size in the range of 0.2 to 5 µm.

6. The membrane according to one of the claims 1 through 5, **characterized by the fact that** the membrane body (4, 4') has a thickness in the range from 100 to 500 µm.

7. The membrane according to claim 6, characterized by the fact that the membrane body (4, 4') has a thickness in the range from 200 to 300 µm.

8. The membrane according to one of the claim 1 through 7, **characterized by the fact that** water was extensively removed from the membrane body (4, 4') after the coupling in a drying stage.

9. The membrane according to one of the claim 1 through 7, **characterized by the fact that** the membrane body (4, 4') is impregnated with glycerine.

10. A filtration module for separation of biomolecules from a liquid, comprising a housing and at least one membrane, **characterized by the fact that** the membrane (3, 3', 3") is constructed according to one of the claims 1 through 9.

11. The filtration module according to claim 10, **characterized by the fact that** a plurality of membranes (3") is arranged in several layers in the housing (2").

12. The filtration module according to claim 10 or 11, **characterized by the fact that** a plurality of membranes (3') is arranged in series in the housing (2').

13. A method for separating biomolecules from a liquid by means of membranes having microporous membrane bodies to which affinity ligands, capable of interaction with the molecules to be removed, are coupled, **characterized by the fact** that the following steps are carried out:

a) Coupling of an affinity ligand in a solution means to the membrane body (4, 4'),

b) washing of the membrane body (4, 4') with at least one washing medium,

c) extensive removal of the last washing medium with a washing procedure,

d) intermediate dry storage of the membranes (3, 3', 3") when this is required, and

e) filtration of the liquid through the membranes (3, 3', 3") so that the biomolecules are removed.

14. The method according to claim 14, **characterized by the fact** that in connection with step b), the following step is introduced:

b1) A strongly volatile organic component miscible with the washing medium is added to the washing medium at the end of the washing according to step b).

15. The method according to claim 14, characterized by the fact that glycerine is added as a strongly volatile organic component.

Figure 1 ~ Figure 3